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Investigation of the expression of the HAESA receptor-like kinase as regulated by the STM and ATH1 homeodomain transcription factors in Arabidopsis thaliana

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Investigation of the expression of the *HAESA* receptor-like kinase as regulated by the STM and ATH1 homeodomain transcription factors in *Arabidopsis thaliana*

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ABSTRACT
DARBY O'GEIL RAYBOURN: Regulation of *HAESA* by STM and ATH1
(Under the direction of Dr. Sarah Liljegren)

Abscission, the shedding of plant organs, is a key process in plant development. Abscission takes place in highly regulated regions called abscission zones (AZ). ARABIDOPSIS THALIANA HOMEOBOX GENE1 (ATH1) and SHOOT MERISTEMLESS (STM) are organ boundary transcription factors that have previously been observed to play a role in sepal-stem boundary formation. Plants carrying mutations in both genes have flowers in which the sepal-stem boundary is abolished. The double mutant flowers are also observed to retain all floral organs. Because abscission zones in *Arabidopsis* form at organ boundary regions, we hypothesized that the STM and ATH1 transcription factors play a role in abscission zone differentiation. An important gene known to be expressed in floral organ AZs is *HAESA* (*HAE*). HAE is a transmembrane receptor kinase that activates the MAP kinase signaling cascade required for cell separation. As a first test of our hypothesis that the STM and ATH1 transcription factors are involved in organ abscission, I analyzed the expression of *HAE* in wild-type and in *stm ath1* double mutant plants. This experiment was conducted using a *HAE:GUS* transgene. In my results I found that in *stm ath1* double mutant plants expression of *HAE* was substantially reduced in the regions where the floral organ abscission zones would normally form. My results provide the first molecular evidence that the ATH1 and STM transcription factors are critical for the regulation of *HAESA* expression and the process of abscission zone signaling.

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LIST OF ABBREVIATIONS

ATH1	ARABIDOPSIS THALIANA HOMEOBOX GENE1
AZ	abscission zone
FAA	Formalin-Acetic Acid-Alcohol
FLC	FLOWERING LOCUS C
GUS	β- glucuronidase
HAE	HAESA
MAP	Mitogen-activated protein
MAPK	MAP kinase
MKK4	MAP kinase kinase 4
MKK5	MAP kinase kinase 5
RLK	receptor-like protein kinase
SAM	shoot apical meristem
STM	SHOOT MERISTEMLESS
W343*	Tryptophan at position 343 changed to a stop codon
WT	wild-type
X-Gluc	5-bromo-4-chlor-3-indolyl-beta-D-glucuronnic acid
Y399*	Tyrosine at position 399 changed to a stop codon

1. INTRODUCTION:

Abscission, a key process in plant development, is characterized by the shedding of plant organs such as leaves, fruits, and flowers. The abscission process is most commonly observed in deciduous forests across the globe by the shedding of their leaves. This phenomenon can also be observed in the model organism *Arabidopsis thaliana* (Jinn et al., 1999). Abscission occurs by hydrolysis of the middle lamella of a specialized cell layer (Cho et al., 2008). Abscission takes place in designated abscission zones (AZ) that are usually associated with organ boundaries. In order for hydrolysis to take place, specialized cells and hydrolyzing enzymes must be present in these zones (Cho et al., 2008). In *Arabidopsis,* AZs are characterized by small, cytoplasmically dense cells typically two to six layers deep located at the base of floral organs (Cho et al., 2008). Exogenous ethylene, a chemical that promotes cell separation, is required for abscission in *Arabidopsis* (Cho et al., 2008). Development of these zones and the entire abscission process are highly regulated. While components of the abscission process in *Arabidopsis* have been identified, there is much that remains unknown.

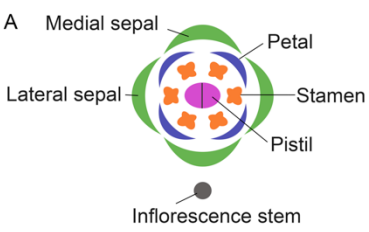

Figure 1. Diagram of the floral organs present in *Arabidopsis thaliana* flower.

It has been previously found that two homeobox genes *ARABIDOPSIS THALIANA HOMEOBOX GENE1 (ATH1)* and *SHOOT MERISTEMLESS (STM)* are involved in organ abscission (Gómez-Mena et al., 2008, Long et al., 1996). Homeobox genes encode homeodomain transcription factors, which are proteins with a DNA-binding domain that allows them to activate or repress gene expression (Bürglin et al., 2015). Both *ATH1* and *STM* are transcription factors (Gómez-Mena et al., 2008, Long et al., 1996). The DNA-binding domain of transcription factors is composed of 60 amino acids and is known as a homeodomain (Mukherjee et al., 2009). The homeodomain folds into three alpha-helices that bind to conserved recognition sequences located on DNA (Mukherjee et al., 2009). These recognition sequences are called consensus sequences.

ATH1 encodes for a BELL-type homeodomain transcription factor. This protein is characterized as a light-regulated transcription factor required for development of the basal boundaries of shoot organs (Gómez-Mena et al., 2008). By observing plants with a homozygous *ath1* mutation known as *ath1-3*, the role of *ATH1* in the AZ region was determined. Visual observations of *ath1-3* plants showed that stamens were not shed after fertilization. Cross sections through these same plants showed that small cells

characteristic of the abscission zone region were absent in *ath1-3* plants. Additionally, *ath1-3* plants showed partial fusion at the base of the stamens. Researchers confirmed the presence of *ATH1* in the basal region of *Arabidopsis* flowers using the *BP:GUS* marker gene. *BREVIPEDICULLUS (BP)* is known to be strongly expressed in this basal region. Diminishing expression of *BP:GUS* in this region of *ath1-3* plants was observed when compared to wild-type plants. These observations confirm that *ATH1* plays a crucial role in the development of abscission zone boundaries (Gómez-Mena et al., 2008).

STM codes for a class I KNOTTED-like transcription factor (Long et al., 1996). The KNOTTED class of homeodomain proteins takes part in shoot apical meristem (SAM) formation. Most recessive mutations in *STM* occur upstream of the homeodomain, truncating the protein and causing an absence of a SAM (Long et al., 1996). Based on this knowledge that researchers concluded that the protein encoded by *STM* is an *Arabidopsis KNOTTED* homologue and is expected to promote SAM formation.

ATH1 has previously exhibited heterodimerization with *STM*, indicating that *ATH1* and *STM* function together (Gómez-Mena et al., 2008). *STM* lacks an efficient nuclear localization signal (NLS) (Cole et al., 2006). An NLS is necessary for a protein to move from the cytoplasm into the cell nucleus. Due to an absence of this important signal *STM* is primarily located in the cytoplasm (Rutjens et al., 2009). *ATH1*, however, does contain an efficient NLS and is therefore found in both the cytoplasm and the nucleus (Rutjens et al., 2009). Because *STM* requires *ATH1* for relocalization, *STM* and *ATH1* heterodimerize. This means *STM* and *ATH1* are interdependent for nuclear localization and consequently have overlapping functions (Rutjens et al., 2009). Evidence that *ATH1*

and STM are heterodimers suggests that STM is also important in organ boundary formation.

Previously, the Liljegren carried out a screen looking for *Arabidopsis* mutants with altered organ abscission. Two mutants were identified in which the stamens were retained even after fertilization. Evidence of the retained stamens can be observed in Figure 2B, C. These mutants were found to affect the STM and ATH1 transcription factors. Double mutant flowers containing mutations in both transcription factors retained all floral organs suggesting that organ abscission has been completely blocked (Figure 2D).

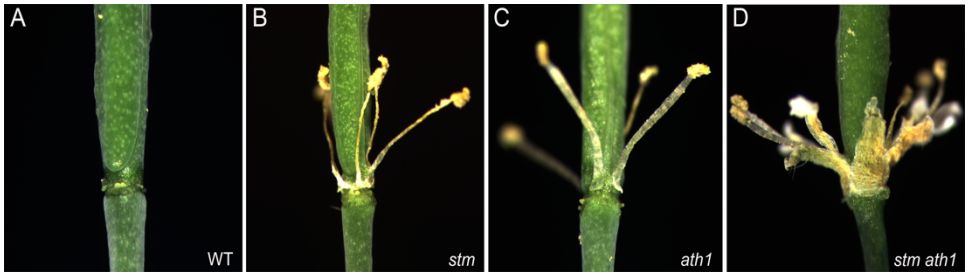

Figure 2. Mutations in *STM* and A*TH1* alter floral organ abscission. (A) Wild-type *Arabidopsis* flowers release their sepals, petals, and stamens after fertilization. **(B, C)** Flowers containing mutations in either *STM* or *ATH1* retain their stamens. **(D)** Flowers with mutations in both *STM* and *ATH1* fail to undergo organ abscission.

As noted above, one mutation identified in our screen is a novel allele of *ATH1* known as *ath1*. The mutation is caused by a splice site error located at amino acid 399 (Y399*) within the homeodomain (Figure 3). The other is a weak allele of *STM* known as *stm*. This mutation is caused by a nonsense mutation that introduces a premature stop codon at amino acid 343 (W343*), which is within the homeodomain (Figure 3).

Figure 3. *STM* and *ATH1* homeobox transcription factor mutations occur in their homeodomain regions. Each light blue area labeled "homeodomain" denotes the DNA binding domains of this family of transcription factors**.**

Scanning electron micrograph images of the *ath1* single mutant and *stm ath1*double mutant flowers were previously taken by this lab and show evidence of altered sepal-pedicel boundaries (Figure 4). Evidence that stm ath1 double mutant flowers retain all floral organs means that organ abscission has been altered. Evidence that the sepal-receptacle boundary has been completely obscured means that STM and ATH1 transcription factors may affect abscission at an early stage of development. Fusion of the sepals in this region prevents organ abscission and causes the plant to retain its stamens. Studies of *ath1, stm, and ath1 stm* plants can provide insight into the proteins that these genes encode and how they are involved in other essential parts of plant development.

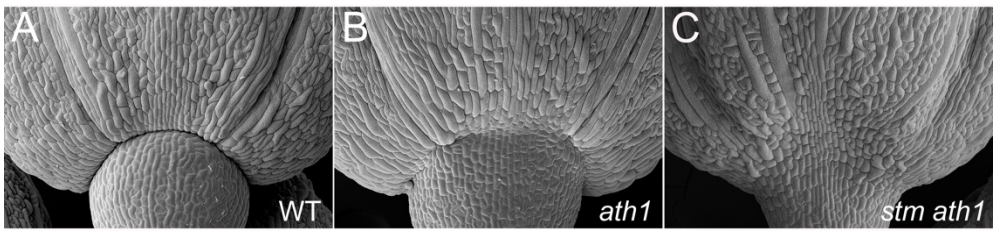

Figure 4. The sepal-stem boundary is abolished in *stm ath1* flowers. Scanning electron micrographs of a wild-type and mutant flowers. (A) A well-defined boundary is formed between the sepals and stem of wild-type flowers. (B) In *ath1* single mutant flowers, this organ boundary is less distinct. (C) In *ath1 stm* double mutant flowers, this boundary is absent.

My research focuses specifically on *HAESA (HAE)*, a leucine-rich repeat receptor-like protein kinase (RLK). Kinases are enzymes that phosphorylate other proteins to either activate or deactivate them. *HAESA (HAE)* was one of the first *Arabidopsis* RLKs to be identified and is known to be expressed in floral organ AZs (Cho et al., 2008). For this reason, *HAE* is considered to be a marker of floral organ abscission zones. *HAE* has previously been shown to play a vital role in organ abscission and is believed to be a part of a MAPK signaling cascade involved with abscission regulation (Cho et al., 2008). Previous research has shown *HAE, HAESA-like 2 (HSL2)*, and *Inflorescence Deficient in Abscission (IDA)* all have similar functions in floral organ abscission in *Arabidopsis,* and that loss of function mutations in these genes results in abscission-defective phenotypes (Cho et al., 2008). However, experiments created to gain further knowledge of the signaling pathway involved in abscission show *MKK4* and *MKK5*, two MAP kinase kinases MAP kinase (MPK), can rescue the mutant phenotypes and allow normal floral organ abscission to proceed (Cho et al., 2008). These findings led researchers to believe these genes may be located downstream of *IDA*, *HAE*, and *HSL2* in

a signaling pathway. This research allowed for a better understanding of the vital role that *HAE* plays in this organ abscission pathway, and made it possible for this lab to use *HAE* expression at the sepal-pedicel boundary region as an observable and comparable marker.

A GUS reporter system can be used to observe the expression of the *HAE* promoter. GUS is a reporter gene that comes from the bacteria *Escherichia coli*, which encodes for the enzyme β-glucuronidase. Because *HAE* is involved in floral organ abscission, the *HAE:GUS* marker is a known maker for Arabidopsis floral organ abscission zone cells (Jinn et al., 1999). In order to observe *HAE* expression in *Arabidopsis,* we observed GUS staining patterns in the sepal-pedicel region of stage 12-14 flowers.

Our lab's previous results have found morphological evidence that *STM* and *ATH1* play a critical role in establishing the sepal-pedicel boundary (Figure 4). With the knowledge of *HAESA*'s role in organ abscission, I have hypothesized that the regulation of genes that are critical for abscission zone function are altered in *ath1* and *stm* flowers. As a first test of this hypothesis, I plan to look specifically at the regulation of *HAESA* and the effect that *STM* and *ATH1* have on the regulation of *HAESA*. These results would be evident in comparison with mutant plant abscission zone formation. Observation of *HAE* expression allows for molecular evidence of the importance of *ATH1* and *STM* activity in regulation of key genes involved in abscission zone signaling. By crossing and growing *ath1 HAE-GUS, stm HAE-GUS,* and *ath1 stm HAE-GUS* the properties of organ abscission genes can be further studied. By observing and analyzing staining patterns one can deduce information about gene properties. These results can be used to further

understand the involvement of organ boundary genes, ATH1 and STM, in establishing sepal boundaries and abscission zone signaling.

2. MATERIALS/ METHODS:

I. Plant materials and growth conditions

The Landsberg *erecta* (L*er*) ecotype of *Arabidopsis thaliana* was used as the wild-type. The L*er* ecotype was also used for the background for all mutant plants. The growth conditions were sixteen hours light and eight hours dark at 21°C and 50% humidity. The plants were watered every other day, alternating between water and Miracle Grow (200 ppm). The soil used was Metromix 350.

II. Histochemical GUS Analyses

A transgenic *HAE:GUS* line was crossed into the *stm* and *ath1* mutants to generate single and double mutants carrying this abscission zone maker (Leslie et al., 2010). GUS reporter system analyses were performed using inflorescences (clusters) of wild-type and mutant flowers. The tissue was harvested and fixed in cold 90% acetone for 20 minutes. The fixed tissue was then rinsed in a 50 mM $NaPO_4$ pH 7.2 buffer, 0.5 mM potassium ferrocyanide, and 0.5 mM potassium ferricyanide solution. The tissue sat in the rinse solution for 5 minutes while shaken. The tissue was then stained in a 50 mM $NaPO_4$ pH 7.2 buffer, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 2 mM X-Gluc solution. The tissue was incubated for a minimum of 16 hours. at 37°C. After 16 hours, the staining solution was removed and followed with ethanol washes of 15% and 30%, for 30 minutes each respectively. The stained tissues were observed under a dissecting microscope and then stored in 50% ethanol solution at 4°C until final tissue fixation.

GUS stained inflorescences were fixed in a 5% formaldehyde, 50% ethanol, and 10% acetic acid solution for preservation until further use. Each tissue sample was put in a separate vital containing a total of 10mL of fixation solution for 1 hour. After a rinse of 70% ethanol, the tissue was put in 70% ethanol for long term storage in a 4°C fridge.

III. Plant Genotyping

DNA was prepared from leaves of mutant plants using the Plant DNeasy© Plant Mini Kit (QIAGEN©). Tissue samples were disrupted using a tissue pulverizer. 400 μL of Buffer AP1 for lipid breakdown and 4 μL of RNase A for RNA breakdown were added to the tissue samples, then each sample was vortexed and incubated for 10 minutes at 65°C. 130 μL of Buffer P3 for neutralization was then added, mixed, and incubated for 5 minutes on ice. After incubation, the lysate was centrifuged for 5 minutes at 14000 rpm. The lysate was pipetted into a QlAshredder spin column placed in a 2 mL collection tube. The lysate was centrifuged for 2 minutes at 14000 rpm. Flow-through was transferred into a new tube without disturbing the pellet. 1.5 volumes of Buffer AW1 for protein denaturing was added and mixed by pipetting. 650 μL of the mixture was transferred into a DNeasy© Mini spin column placed in a 2 mL collection tube. The mixture was centrifuged for 1 minutes at 8000 rpm. Flow-through was removed and discarded, and the process repeated with the remaining sample. The spin column was placed in a new 2 mL collection tube and 500 μL of Buffer AW2 for salt removal and purification was added. The mixture was centrifuged for 1 minute at 8000 rpm and flow-through discarded. Another 500 μL of Buffer AW2 was added and the mixture was centrifuged for 2 minutes at 14000 rpm. The spin column was transferred to a new 2 mL microcentrifuge tube. 100 μL Buffer AE was added for elution and incubated for 5 minutes at room temperature and

then centrifuged for 1 minute at 8000 rpm. The step was repeated. The plant genomic DNA was stored at -20°C.

In order to genotype the *stm* and *ath1* mutant plants, the Polymerase Chain Reaction (PCR) was used to amplify the *STM* and *ATH1* gene regions using mutant genomic DNA as a template. The oligos that were used to amplify these regions are described in Table 1. A master mixture was created using a per reaction ratio of 1x Standard Taq Reaction Buffer #B9014S (NEW ENGLAND *BioLabs)*, 1.5 mM $MgCl_2$ (Apex), 0.25 mM dNTP (PROMEGA), 0.7 μM forward and reverse primer (Table 1), 0.25 U/μL Taq DNA Polymerase #M0273S (NEW ENGLAND *BioLabs*), and 13 μL deionized H_2O. 20 μL samples of PCR solution were made containing 18 μL of the master mixture combined with 2 μL of genomic DNA. The samples ran on either a *STM* PCR cycle (Table 2) or an *ATH1* PCR cycle (Table 3).

Next, in order to improve the quality of the ATH1 restriction digest the PCR products were desalted via ethanol precipitation. This step was added due to high salt content causing a deactivation of the MluCI enzyme. First, 60 μL of 100% ethanol (stored at -20°C) and 2.1 μL sodium acetate at pH 5.2 at 4°C was added. The sample was allowed to sit overnight at -20°C. The sample was spun down at 4°C at 15000 rpm for 45 minutes. The ethanol supernatant was removed leaving the pellet undisturbed. After, 70% ethanol (stored at -20°C) was then added. The sample was spun down at 4°C at 15000 rpm for 30 minutes. Ethanol was removed leaving the pellet undisturbed. The sample was put in a 37°C water bath to evaporate remaining ethanol without drying out the pellet. The pellet was resuspended in 20 μL ddH_2O.

The DNA samples were digested. Plants homozygous for *stm* were distinguished based on a BsrI restriction site present in the wild-type allele of the *STM* gene (Figure 5A). Plants homozygous for *ath1* were distinguished based on a MluCI restriction site present in the mutant allele of the *ATH1* gene (Figure 5B). *STM* PCR products were digested using a BsrI #R0527S (NEW ENGLAND *BioLabs*) restriction enzyme in the recommended enzyme buffer, NEBuffer 3.1#B7203S (NEW ENGLAND *BioLabs*); a 20 ul (3 ul master mix, 17 ul PCR) sample was incubated at 65°C for 4 hours. *ATH1* PCR products were digest using a MluCI #R0538S (NEW ENGLAND *BioLabs*) restriction enzyme in the recommended enzyme buffer, CutSmart™ Buffer (NEW ENGLAND *BioLabs*). A 20 ul (3 ul master mix, 17 ul PCR) sample was incubated at 37°C for 3 hours.

After completion of digests, DNA samples were analyzed using gel electrophoresis. A 3% agarose gel was used for observing *stm* digests and a 1% agarose gel was used for observing *ath1* digests. 3 μL of loading dye was added to each digest sample. A 13 μL sample of *ath1* was loaded onto 1% gel. A 15 μL sample of *stm* was loaded onto 3% gel. Gels ran at 100V and ~175 mA.

IV. Plant Imaging

Digital images were taken with a Stemi SV11 dissecting microscope and Axiocam HR camera (Carl Zeiss, Germany) or PowerShot SX160 IS (Canon, Melville, NY). Image brightness and contrast were adjusted with Photoshop CS6 (Adobe, Mountain View, CA).

Sequence Name	Sequence 5’ to 3’
ATH1gt1F	GGATGTTCCAAAACTTCCTTCACCC
ATH1gt2R	GCTTGATTTTTTCCTAGCCCTAATCTC
STAMgtF	GTTCATAAACCAGAGGAAACGGCACTG
STAMgtR	GAGGAGATGTGATCCATTGGGAAAGG

Table 1. *ATH1* and *STM* oligos sequences.

Step	Temperature (ºF)	Time (Minutes)
1	94.0	4:00
2	94.0	0:30
3	54.0	0:30
4	72.0	0:30
5	*Repeat *Step 2* 30 Times	*Repeat *Step 2* 30 Times
6	4.0	Forever

Table 2. *STM* PCR conditions.

Step	Temperature (ºF)	Time (Minutes)
1	94.0	4:00
2	94.0	0:30
3	55.0	0:30
4	72.0	0:30
5	*Repeat *Step 2* 30 Times	*Repeat *Step 2* 30 Times
6	4.0	Forever

Table 3. *ATH1* PCR conditions.

3. RESULTS:

Expression of *HAE:GUS* marker is altered in *stm ath1* flowers

To observe the expression of the receptor-like kinase gene, *HAESA*, in the abscission zones of *Arabidopsis* flowers we crossed a *HAE:GUS* transgenic plant with an *ath1* plant, a *stm* plant, and a *stm ath1* double mutant plant. In order to identify the genotype of the mutant plants we performed PCR, restriction digests, and gel electrophoresis on plant genomic DNA (Figure 5).

Histochemical staining was used to confirm the presence of the *HAE:GUS* transgene. The β- glucuronidase (GUS) gene encodes an enzyme that cleaves the X-Gluc substrate forming a blue precipitate which can be observed (Figure 6, 7, 8). In plants that carry the *HAE:GUS,* transgene expression of GUS is controlled by the *HAE* promoter (Jinn et al., 1999). After isolating and confirming single and double mutant plants containing the transgene, we observed and compared *HAE* expression in the samples. Observation of wild-type plants showed expression of the *HAE:GUS* marker in the abscission zone cells at the sepal-pedicel boundary of each flower (Figure 6A, 7A, 8A). We found that *stm* and *ath1* single mutant plants also had *HAE:GUS* expression at the sepal-pedicel boundary and associated abscission zone cells (Figure 6B-C, 7B-C, 8B-C). However, in the *stm ath1* double mutant plants we discovered that there is a substantial reduction of *HAE*:*GUS* expression in the sepal-pedicel region (Figure 6D, 7D, 8D). These results indicate that expression of the *HAE:GUS* marker in *Arabidopsis* abscission zone cells is dependent on the activity of the STM and ATH1 transcription factor

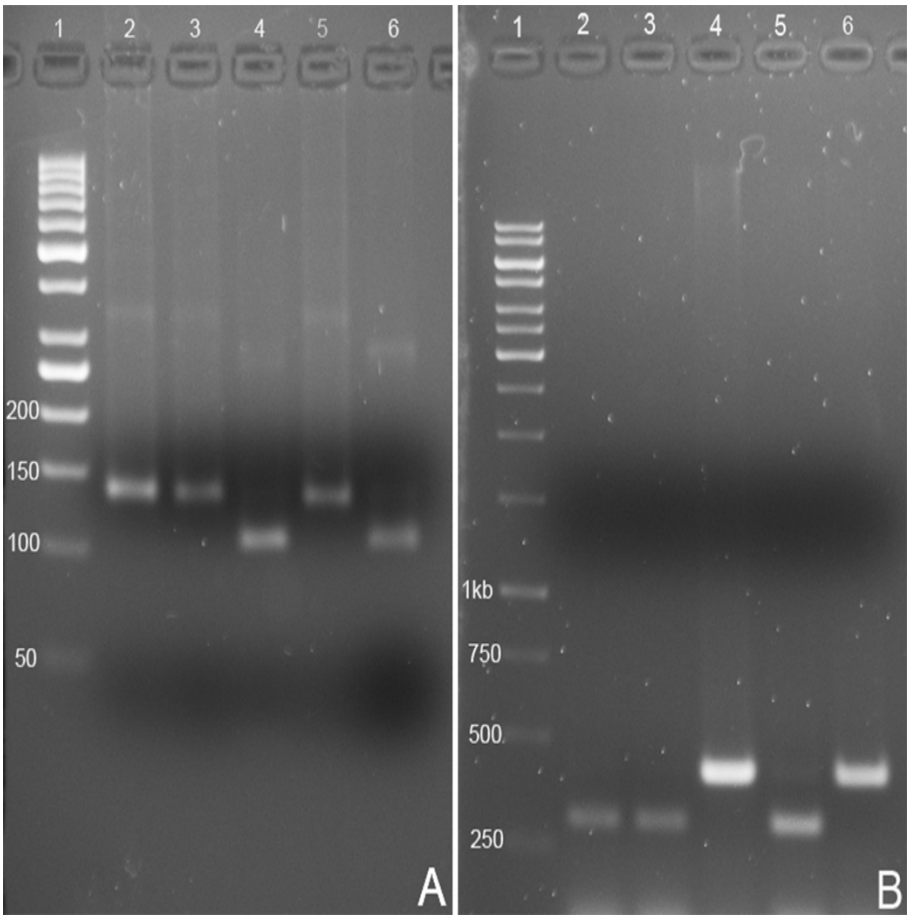

Figure 5. Genotyping analysis of the *stm*, *ath1*, and *stm ath1* mutants. (A) A 3% agarose gel is shown with the BsrI enzyme digested PCR products used to determine the *stm* homozygous, heterozygous**, and WT genotypes. Lane 1 is a 50 base pair (bp) ladder with sizes to the left. The BsrI enzyme digests the WT PCR product into two segments (106 bp, 29 bp)*. The *stm* mutant PCR product is left undigested (135 bp). Based on the controls in Lane 5 (*stm* mutant) and 6 (WT), Lane 2 and 3 are *stm* mutant flowers, and Lane 4 is a WT flower. **(B)** A 1% agarose gel is shown with the MluCI enzyme digested PCR products used to determine the *ath1* homozygous, heterozygous, and WT genotypes. Lane 1 is a 1 kilobase pair ladder with sizes to the left in bp. The MluCI enzyme digests the *ath1* mutant PCR product into three segments (306 bp, 158 bp, 115 bp)*. The WT PCR product is left undigested (421 bp). Based on the controls in Lane 5 (*ath1*mutant) and 6 (WT), Lane 2 and 3 are *ath1* mutant flowers, and Lane 4 is a WT flower.

* Small digest segments tend to run off of the gel. (29 bp on A/ 158 bp, 115 bp on B)

** None of the samples shown here were heterozygous mutants. Heterozygous mutants can be detected when both mutant and WT size bands are observed.

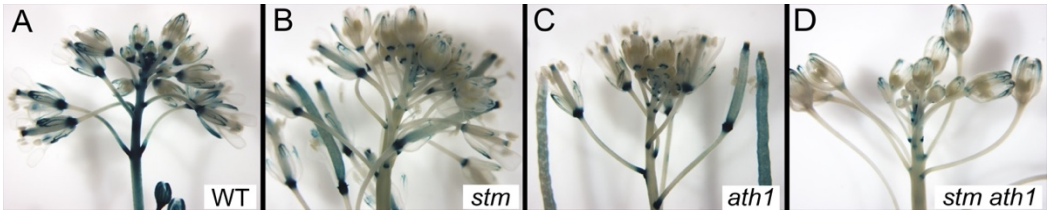

Figure 6. Expression of the *HAE:GUS* marker is substantially reduced in *stm ath1* flowers. Images of wild-type and mutant inflorescences. **(A)** In wild-type plants, expression of the *HAESA* receptor-like kinase is found in the abscission zone cells at the sepal-pedicel region of each flower. **(B, C)** In *stm* and *ath1*single mutant plants *HAE:GUS* expression is still observed at the sepal-pedicel boundaries. **(D)** In *stm ath1* double mutant plants *HAE:GUS* expression in this region is significantly reduced.

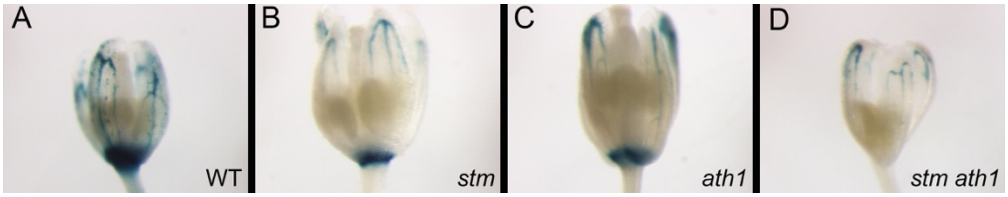

Figure 7. Expression of the *HAE:GUS* marker is substantially reduced in stage 12-13 *stm ath1* flowers. (A) In the stage 12-13 wild-type bud, expression of the *HAESA* receptor-like kinase is found in the abscission zone cells of the sepal-pedicel region. **(B, C)** In the *stm* and *ath1*single mutant buds *HAE:GUS* expression in this region is still observed. **(D)** In the *stm ath1* double mutant bud *HAE:GUS* expression in this region is significantly reduced.

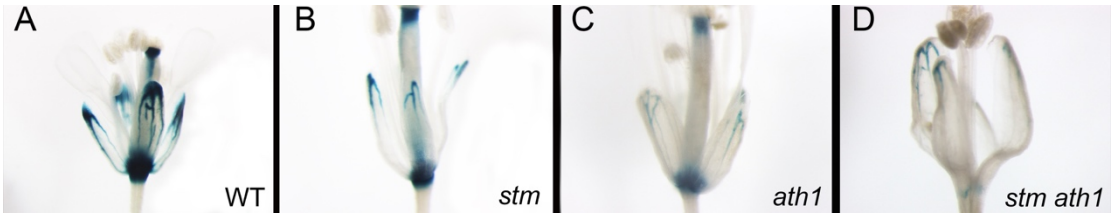

Figure 8. Expression of the *HAE:GUS* marker is substantially reduced in stage 14 *stm ath1* flowers. (A) In the stage 14 wild-type bud, expression of the *HAESA* receptor-like kinase is found in the abscission zone cells of the sepal-pedicel region. **(B, C)** In the *stm* and *ath1*single mutant buds *HAE:GUS* expression in this region is still observed. **(D)** In the *stm ath1* double mutant bud *HAE:GUS* expression in this region is significantly reduced.

4. DISCUSSION:

As a result of previous research and our understanding of abscission zone regulation, we hypothesized that transcription factors *ATH1* and *STM* are involved in the regulation pathway of abscission zone signaling, specifically by their regulation of *HAESA* receptor-like kinase expression in this region. Previous research in this lab provided visual evidence of *ATH1* and *STM* involvement in sepal-pedicel boundary formation (Figure 5). By completing the experiments outlined in this research, our work now provides molecular evidence of the necessary roles *ATH1* and *STM* play in abscission zone signaling.

Using the transgene *HAE:GUS* as a marker we were able to observe the regulation of *HAESA* by *ATH1* and *STM*. The staining patterns observed in *stm ath1* double mutant transgenic plants show evidence of substantial reduction of *HAE:GUS* in the sepal-pedicel region when both *STM* and *ATH1* are nonfunctional. Reduction of the *HAE:GUS* marker strongly suggests that *HAE* expression requires the activity of *STM* and *ATH* in this region. Furthermore, our observations of sustained *HAE* expression in single mutant flowers indicates that heterodimers *ATH1* and *STM* may act redundantly in the regulation pathway of *HAE*. Our work provides key findings about the gene pathway involved in the floral organ abscission zone formation.

In conclusion, my results found crucial evidence of *HAE* regulation by *ATH1* and *STM*. Evidence provided by my research using the *HAE:GUS* marker suggests that

follow-up studies to further characterize and analyze the expression of *HAE* in *stm ath1* double mutants are warranted. GUS staining provided by my work shows indirect evidence of what regulatory regions are affected by *ATH1* and *STM.* Subsequent research should be carried out as a way to qualitatively and quantitatively analyze *HAE* expression in double mutant flowers and provide a more direct look at *HAE* RNA.

Reverse transcriptase PCR (RT-PCR) and RNA in situ hybridization are two methods that can be used to analyze direct gene expression. RT-PCR is used to qualitatively detect gene expression by creating complementary DNA (cDNA) transcripts from RNA. RT-PCR uses reverse transcriptase to reverse transcribe RNA into its DNA complement. RT-PCR can detect very low levels of RNA because the cDNA complement is very stable and is thus useful in qualitatively looking at the most minimal gene expression. RNA in situ hybridization is used to quantitatively measure a specific RNA sequence in a section of tissue (in situ) using a fluorescent reporter probe.

Another direction for future study is RNA sequencing of wild-type and *stm ath1* flowers. RNA sequencing involves creating a complete cDNA library of mutant and wild-type genomes. By analyzing and comparing these cDNA libraries we can characterize the transcriptomes of the wild-type and mutant flowers and identify any additional downstream targets of *STM* and *ATH1* transcription factors. This would allow for a more complete understanding of the gene pathway involved in organ abscission in *Arabidopsis thaliana.* (Conesa et al., 2016).

RNA sequencing is necessary because, although we have evidence of *ATH1* and *STM* contribution to abscission zone regulation, the position of *ATH1* and *STM* in the signaling pathway is still unclear (Figure 9). One explanation would be that *ATH1* and

STM affect *HAE* by direct regulation of the *HAE* promoter (Figure 9A). Another explanation would that *ATH1* and *STM* affect *HAE* by indirect regulation, affecting other genes in the signaling pathway upstream of *HAE* (Figure 9B). This lab has not yet completed an analysis of the *HAE* promoter that would allow observation of possible transcription factor consensus sequences.

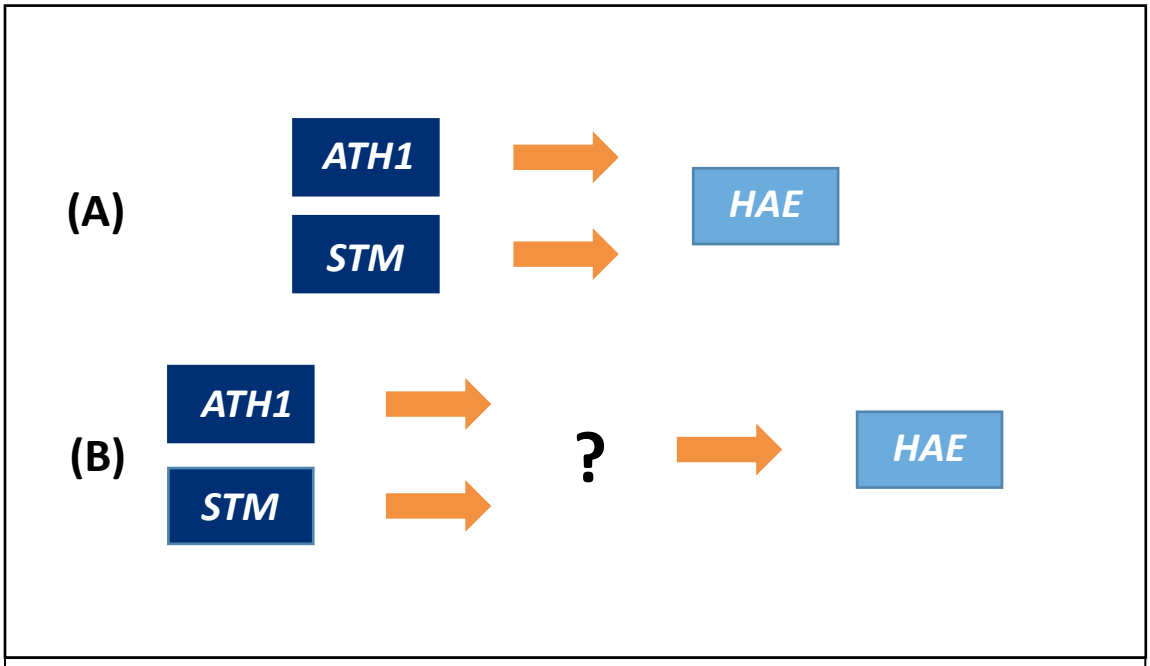

Figure 9. *HAE* could be a direct or indirect target of the STM and ATH1 transcription factors. (A) STM and ATH1 may directly bind to the promoter and activate expression of *HAE*. **(B)** STM and ATH1 may directly regulate other unknown transcription factors (indicated here by "?"), which then directly regulate expression of *HAE*.

Results of this research also indicate that there may be opportunities for follow up studies to be completed in order to determine the individual effects ATH1 and STM have on the expression of *HAE*. In our research, subtle changes in staining patterns of single mutant transgenic plants suggest that ATH1 and STM may have individual effects on the expression of *HAE*. However, our results were insufficient to report any real qualitative data due to a small sample size ($n < 5$). In the future a larger sample size ($n > 5$), as well

as control over the number of *HAE:GUS* transgene insertions in the plant, could be used to observe these effects.

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